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APPLICATION

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TITLE: **NOVEL PLASMIDS AND UTILIZATION THEREOF**

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DESCRIPTION

NOVEL PLASMIDS AND UTILIZATION THEREOF

Technical Field

[0001] The present invention relates to novel plasmids derived from any of microorganisms belonging to the genus *Rhodococcus* (hereinafter referred to as "the genus *Rhodococcus*") and to utilization thereof. More specifically, the invention relates to plasmids or their partial DNA fragments (hereinafter also referred to simply as "DNA fragments"), and to shuttle vectors, vectors, transformants, aminoketone asymmetric reductase production methods and optically active aminoalcohol production methods which utilize them.

Background Art

[0002] The genus *Rhodococcus* is known to produce enzymes involved in nitrile metabolism and to produce enzymes which asymmetrically reduce aminoketones. In particular, *Rhodococcus erythropolis* is known to have very high aminoketone asymmetric reduction activity. Such microorganisms and enzymes act on α-aminoketones to high selectively produce optically active β-aminoalcohols at high yields (for example, Patent documents 1 and 5). Thus, it has long been desired to develop a host-vector system intended for mass production of useful enzymes and the like in the genus *Rhodococcus*. However, the development of vectors suitable for the genus *Rhodococcus* as hosts has lagged behind. Only a few strains of the genus *Rhodococcus* have been found with plasmids, namely *Rhodococcus* sp. H13-A (Non-patent document 1), *Rhodococcus rhodochrous* ATCC4276 (Patent document 2), *Rhodococcus*

rhodochrous ATCC4001 (Patent document 3) and *Rhodococcus erythropolis* IFO12320 (Patent document 4).

[0003] [Patent document 1] WO01/73100

[Patent document 2] Japanese Unexamined Patent Publication HEI No. 4-148685

[Patent document 3] Japanese Unexamined Patent Publication HEI No. 4-330287

[Patent document 4] Japanese Unexamined Patent Publication HEI No. 9-28379

[Patent document 5] WO02/070714

[Non-patent document 1] J. Bacteriol., 170, 638, 1988

Disclosure of the Invention

Problems to be Solved by the Invention

[0004] As mentioned above, it has been desired to develop new vectors for breeding and improve to industrially useful strains (mutant strains) from the genus *Rhodococcus*. In particular, self-cloning systems are preferred from the standpoint of safety of the recombinant DNA microbes and their products which may be used as foods and additives. It is an object of the present invention to provide novel plasmids that can be used as vectors for such a host-vector system.

[0005] It is desirable to create recombinant microbes suitable for industrial application from among *Rhodococcus erythropolis* which has aminoketone asymmetric reduction activity. In particular, it is a first object of the invention to provide novel plasmids or their partial DNA fragments which can be used to create such recombinant microbes.

[0006] If a plasmid such as described above can be obtained, it

would become easy to construct a shuttle vector that is replicable even in other microbes. It is therefore a second object of the invention to provide nucleotide sequence data relating to DNA replication (replication region, etc.) necessary for construction of such a shuttle vector.

[0007] It is a third object of the invention to provide shuttle vectors that are replicable in both the genus *Rhodococcus* and *E. coli*.

[0008] It is a fourth object of the invention to apply the shuttle vectors to an aminoketone asymmetric reductase.

Means for Solving the Problems

[0009] The present inventors carefully screened plasmids for vector construction from among *Rhodococcus* strains, and as a result discovered several novel plasmids usable as vectors for host-vector systems.

[0010] Furthermore, the present inventors found that it is possible to construct shuttle vectors by transferring into the aforementioned plasmids a drug resistance gene and a gene region that is replicable in *E. coli*. As a result there were obtained nucleotide sequence data, plasmids and shuttle vectors that achieve the objects stated above, and the present invention has thereupon been completed.

[0011] Specifically, the present invention provides a DNA fragment, a DNA, a plasmid, a shuttle vector, a vector, a transformant, a method for production of an aminoketone asymmetric reductase, and a method for production of an optically active aminoalcohol, according to following (1) to (39).

(1) A DNA fragment having at least one nucleotide sequence selected

from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37.

(2) A plasmid or a partial DNA fragment thereof, characterized by comprising a DNA replication region having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37.

(3) A DNA fragment having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 14, SEQ ID NO: 17 and SEQ ID NO: 22.

(4) A plasmid or a partial DNA fragment thereof, characterized by comprising a coding region for a DNA replication-related protein having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 14, SEQ ID NO: 17 and SEQ ID NO: 22.

(5) A plasmid or a partial DNA fragment thereof, characterized by comprising a coding region for a DNA replication-related protein having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 14, SEQ ID NO: 17 and SEQ ID NO: 22 and comprising a DNA replication region having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37.

(6) A DNA fragment having the nucleotide sequence set forth as SEQ

ID NO: 76.

(7) A plasmid or a partial DNA fragment thereof, characterized by comprising a promoter region having the nucleotide sequence set forth as SEQ ID NO: 76.

(8) A plasmid or a partial DNA fragment thereof, characterized by comprising a coding region for a DNA replication-related protein having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 14, SEQ ID NO: 17 and SEQ ID NO: 22, comprising a DNA replication region having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37, and comprising a promoter region having the nucleotide sequence set forth as SEQ ID NO: 76.

(9) A circular plasmid characterized by comprising a plasmid or a partial DNA fragment according to any one of (1) to (8), wherein the numbers of restriction endonuclease cleavage sites are *Bam*H I: 2, *Eco*R I: 2, *Kpn* I: 1, *Pvu* II: 1, *Sac* I: 1 and *Sma* I: 1, and the size is approximately 5.4 kbp.

(10) A plasmid having the nucleotide sequence set forth as SEQ ID NO: 73.

(11) A plasmid or a DNA fragment according to any one of (1) to (10), characterized by being derived from a bacterium belonging to the genus *Rhodococcus*.

(12) A DNA fragment having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ

ID NO: 70, SEQ ID NO: 71 and SEQ ID NO: 72.

(13) A plasmid or a partial DNA fragment thereof, characterized by comprising a DNA replication region having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 70, SEQ ID NO: 71 and SEQ ID NO: 72.

(14) A DNA fragment having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 61, SEQ ID NO: 62 and SEQ ID NO: 69.

(15) A plasmid or a partial DNA fragment thereof, characterized by comprising a coding region for a DNA replication-related protein having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 61, SEQ ID NO: 62 and SEQ ID NO: 69.

(16) A plasmid or a partial DNA fragment thereof, characterized by comprising a coding region for a DNA replication-related protein having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 61, SEQ ID NO: 62 and SEQ ID NO: 69 and comprising a DNA replication region having at least one nucleotide sequence selected from the group consisting of

the nucleotide sequences set forth as SEQ ID NO: 70, SEQ ID NO: 71 and SEQ ID NO: 72.

(17) A plasmid or a partial DNA fragment thereof, characterized by comprising a coding region for a DNA replication-related protein having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 61, SEQ ID NO: 62 and SEQ ID NO: 69, comprising a DNA replication region having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 70, SEQ ID NO: 71 and SEQ ID NO: 72, and comprising a promoter region having the nucleotide sequence set forth as SEQ ID NO: 76.

(18) A DNA fragment having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 67 and SEQ ID NO: 47.

(19) A plasmid or a partial DNA fragment thereof, characterized by comprising a mobilization protein region having at least one nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 67 and SEQ ID NO: 47.

(20) A DNA fragment having the nucleotide sequence set forth as SEQ ID NO: 75.

(21) A plasmid or a partial DNA fragment thereof, characterized by comprising a mobilization-related region having the nucleotide sequence set forth as SEQ ID NO: 75.

(22) A circular plasmid characterized by comprising a plasmid or DNA

fragment according to any one of (12) to (21), wherein the numbers of restriction endonuclease cleavage sites are *Bam*H I: 2, *Pvu* II: 4, *Sac* I: 3 and *Sma* I: 4, and the size is approximately 5.8 kbp.

(23) A plasmid having the nucleotide sequence set forth as SEQ ID NO: 74.

(24) A plasmid or a DNA fragment according to any one of (12) to (23), characterized by being derived from a bacterium belonging to the genus *Rhodococcus*.

(25) A DNA fragment having the nucleotide sequence set forth as SEQ ID NO: 77.

(26) A DNA fragment characterized by comprising a promoter region having the nucleotide sequence set forth as SEQ ID NO: 77.

(27) A shuttle vector replicable in bacteria belonging to the genus *Rhodococcus* and *E. coli*, and comprising a plasmid or partial DNA fragment thereof according to any one of (1) to (26) and a DNA region replicable in *E. coli*.

(28) A vector characterized by being constructed using a shuttle vector according to (27).

(29) A vector characterized by comprising a plasmid or DNA fragment according to any one of (6), (7), (25) or (26).

(30) A vector according to (28) or (29), characterized by having inserted therein an aminoketone asymmetric reductase gene.

(31) A vector according to (30), characterized in that the aminoketone asymmetric reductase gene is a nucleic acid coding for a protein consisting the amino acid sequence set forth as SEQ ID NO: 78, or a nucleic acid that codes for a protein having the amino acid sequence set

forth as SEQ ID NO: 78 with a deletion, insertion, substitution or addition of one or a plurality of amino acids, and having aminoketone asymmetric reduction activity.

(32) A vector according to (30), characterized in that the aminoketone asymmetric reductase gene is a nucleic acid consisting the nucleotide sequence set forth as SEQ ID NO: 79, or a nucleic acid that hybridizes with nucleic acid having a nucleotide sequence complementary to the nucleotide set forth as SEQ ID NO: 79 under stringent conditions, and that codes for a protein having aminoketone asymmetric reduction activity.

(33) A transformant containing a vector according to (28) or (29).

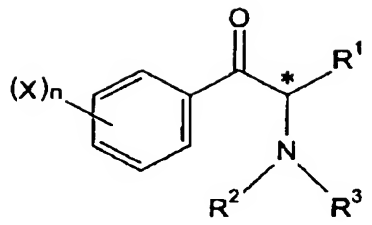
(34) A transformant containing a vector according to any one of (30) to (32).

(35) A method for production of an aminoketone asymmetric reductase, which comprises a culturing step in which a transformant according to (34) is cultured in medium that allows growth of said transformant, and

a purification step in which the aminoketone asymmetric reductase is purified from said transformant obtained in said culturing step.

(36) A method for production of an optically active aminoalcohol, wherein an aminoketone asymmetric reductase obtained by the production method of (35) is reacted with an enantiomeric mixture of an α-aminoketone compound represented by the following general formula (1):

[Chemical Formula 1]



... (1)

wherein X may be the same or different and represents at least one species selected from the group consisting of halogen, lower alkyl, hydroxyl optionally protected with a protecting group, nitro and sulfonyl;

n represents an integer of 0 to 3;

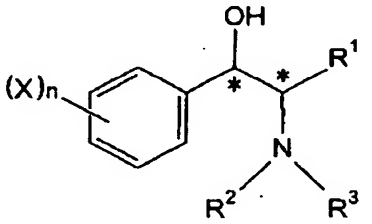
R^1 represents lower alkyl;

R^2 and R^3 may be the same or different and represent at least one species selected from the group consisting of hydrogen and lower alkyl; and

* represents asymmetric carbon,

or a salt thereof, to produce an optically active aminoalcohol compound represented by the following general formula (2):

[Chemical Formula 2]



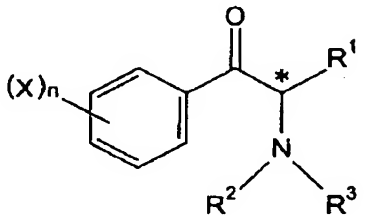
... (2)

wherein X, n, R^1, R^2, R^3 and * have the same definitions as above,

and having the desired optical activity.

(37) A method for production of an optically active aminoalcohol, wherein a transformant according to (34) is reacted with an enantiomeric mixture of an α-aminoketone compound represented by the following general formula (1):

[Chemical Formula 3]



... (1)

wherein X may be the same or different and represents at least one species selected from the group consisting of halogen, lower alkyl, hydroxyl optionally protected with a protecting group, nitro and sulfonyl;

n represents an integer of 0 to 3;

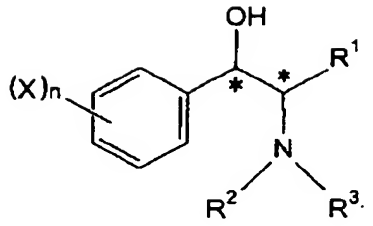
R^1 represents lower alkyl;

R^2 and R^3 may be the same or different and represent at least one species selected from the group consisting of hydrogen and lower alkyl; and

* represents asymmetric carbon,

or a salt thereof, to produce an optically active aminoalcohol compound represented by the following general formula (2):

[Chemical Formula 4]

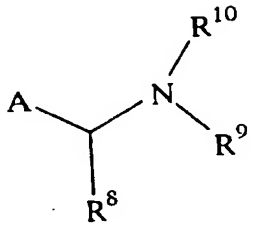


... (2)

wherein X, n, R^1, R^2, R^3 and * have the same definitions as above, and having the desired optical activity.

(38) A production method for an optically active aminoalcohol according to (37), wherein the production method for the optically active aminoalcohol is carried out with further addition of a compound represented by the following general formula (3):

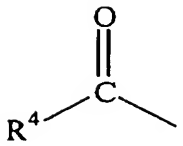
[Chemical Formula 5]



... (3)

wherein A represents the following formula (Y) or (Z):

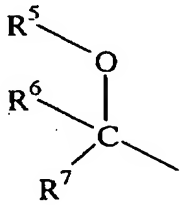
[Chemical Formula 6]



... (Y)

wherein R^4 represents hydrogen, optionally substituted C1-3 alkyl, a C5-10 hydrocarbon ring which is bonded to R^8 or a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^8,

[Chemical Formula 7]



... (Z)

wherein R^5 represents hydrogen, C1-3 alkyl or a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^6 or R^9;

R^6 represents hydrogen, optionally substituted C1-3 alkyl, a C5-10 hydrocarbon ring which is bonded to R^8 or a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^5 or R^9;

R^7 represents hydrogen or optionally substituted C1-6 alkyl;

R^8 represents hydrogen, carboxyl, optionally substituted C1-6 alkyl, a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms

which is bonded to R^4 or a C5-10 hydrocarbon ring which is bonded to R^6;

R^9 represents hydrogen, optionally substituted C1-6 alkyl, optionally substituted C1-6 alkyloxycarbonyl, optionally substituted acyl or a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^5 or R^6; and

R^{10} represents hydrogen or optionally substituted C1-6 alkyl,

or a pharmaceutically acceptable salt or solvate thereof, for production of an optically active aminoalcohol.

(39) A shuttle vector according to (27), having a nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 89 to SEQ ID NO: 100.

Effect of the Invention

[0012] The plasmids of the invention are novel plasmids unknown to the prior art, and are valuable as vectors for host-vector systems belonging to the industrially useful the genus *Rhodococcus*. They are of particular utility in the creation of recombinant microbes capable of industrial asymmetric reduction of aminoketones. An example of asymmetric reduction of an aminoketone to which such microbes may contribute is a reaction for production of d-(1S, 2S)-pseudoephedrine from l-2-methylamino-1-phenyl-1-propanone.

[0013] The plasmids of the invention can coexist in single *Rhodococcus* cell and therefore can be used not only alone for their replicating function, but also as compatible plasmids. That is, by inserting different protein (for example, enzyme) genes into the different plasmids, it is possible to express the proteins simultaneously

in the same cell.

[0014] The shuttle vectors of the invention are useful for creation of industrially useful recombinant microbes of the genus *Rhodococcus* and *Escherichia coli*.

[0015] The nucleotide sequence data relating to DNA replication obtained from the plasmids of the invention may serve as the basis for construction of the aforementioned shuttle vectors, and specifically they provide DNA fragments as constituent elements of the vectors.

Brief Description of the Drawings

[0016]

Fig. 1 is a restriction enzyme cleavage map of plasmid pRET1100.

Fig. 2 is a restriction enzyme cleavage map of plasmid pRET1000.

Fig. 3 is a summary illustration for construction of shuttle vector pRET1101.

Fig. 4 is a summary illustration for construction of shuttle vector pRET1102.

Fig. 5 is a summary illustration for construction of shuttle vector pRET1103.

Best Mode for Carrying Out the Invention

[0017] Preferred embodiments of the invention will now be explained.

[0018] The first plasmid of the invention is a plasmid isolated from the genus *Rhodococcus*, or a derivative thereof. Specifically, it may be isolated from, for example, *Rhodococcus erythropolis*

IAM1400, IAM1503, JCM2893 and JCM2894 strains, has a size of approximately 5.4 kbp and is a circular plasmid cleavable by the restriction enzymes shown in Table 1. The plasmids isolated from each of these strains are designated as pRET1100, pRET1300, pRET1500 and pRET1700, respectively. Plasmids of the invention may be prepared from these sample strains by publicly known methods (for example, boiling, alkali dissolution, cesium chloride density gradient ultracentrifugation: Lab Manual Idenshi Kogaku, 3rd Edition, Chapter 10, pp.55-59, Maruzen).

[0019] [Table 1]

Restriction enzyme	Number of cleavage sites	Fragment sizes (kbp)
*Bam*H I	2	0.4, 5.0
*Eco*R I	2	0.3, 5.1
Kpn I	1	5.4
Pvu II	1	5.4
Sac I	1	5.4
Sma I	1	5.4

[0020] Fig. 1 shows a restriction enzyme cleavage map for pRET1100. This plasmid was sequenced by a publicly known method (using a fluorescent automatic sequencer, for example) and its full nucleotide sequence was revealed to be 5444 bp set forth as SEQ ID NO: 73 of the Sequence Listing.

[0021] The second plasmid of the invention is also a plasmid isolated from the genus *Rhodococcus*, or its derivative. Specifically, it may be isolated from, for example, *Rhodococcus rhodnii* JCM3203, has a size of approximately 5.8 kbp and is a circular plasmid cleavable by the restriction enzymes shown in Table 2. This plasmid is

designated as pRET1000.

[0022] [Table 2]

Restriction enzyme	Number of cleavage sites	Fragment sizes (kbp)
*Bam*H I	2	2.0, 3.8
Pvu II	4	0.1, 1.4, 2.0, 2.3
Sac I	3	0.9, 1.0, 3.9
Sma I	4	0.1, 1.2, 1.6, 2.9

[0023] Fig. 2 shows a restriction enzyme cleavage map for pRET1000. This plasmid was also sequenced by a publicly known method and its full nucleotide sequence was revealed to be 5813 bp set forth as SEQ ID NO: 74 of the Sequence Listing.

[0024] The plasmids of the invention (natural- or wild-types) are circular plasmids that can also be defined by the restriction enzyme cleavage patterns shown in Tables 1 and 2. Thus, the present invention encompasses the following two types of circular plasmids.

(1) A circular plasmid derived from a *Rhodococcus* strain, characterized by having a size of approximately 5.4 kbp and possessing the following restriction enzyme cleavage sites: *Bam*H I:2, *Eco*R I:2, *Kpn* I:1, *Pvu* II:1, *Sac* I:1 and *Sma* I:1.

(2) A circular plasmid derived from a *Rhodococcus* strain, characterized by having a size of approximately 5.8 kbp and possessing the following restriction enzyme cleavage sites: *Bam*H I:2, *Pvu* II:4, *Sac* I:3 and *Sma* I:4.

[0025] As a result of analysis of the nucleotide sequences of plasmids pRET1100 and pRET1000 (i.e., SEQ ID NO: 73 and SEQ ID NO: 74), there is predicted the existence of a group of nucleotide sequences (open reading frames, hereinafter "orf") coding for proteins

for DNA replication or other functions.

[0026] In the relevant technical field, "DNA replication" refers to using DNA itself as template to form two double-stranded DNA molecules exactly identical to existing double-stranded DNA (parent DNA). The replication mechanism consists of three stages: initiation from the starting point of replication (replication origin), DNA chain elongation and termination. During replication, a portion of the DNA double strand is unraveled and new DNA strands are synthesized complementary to each single strand. The double strand is unraveled by DNA helicase and helix destabilizing proteins (also known as single-strand DNA-binding protein), and the unraveled portion is referred to as the replication fork. The template DNA in the direction from 3' to 5' toward the replication fork is the "leading strand", and the one in the direction from 5' to 3' is the "lagging strand". DNA polymerase extends the DNA strand in the direction from 5' to 3'. Therefore when the leading strand is the template, DNA is synthesized in the direction of the replication fork. However when the opposite lagging strand is the template, the DNA strand must be extended in the opposite direction from the replication fork. Consequently, replication of the lagging strand is accomplished in fragments of about 200 bases, known as Okazaki fragments. Every approximately 200 bases, RNA primer is used with DNA as template to synthesize 10 bases of RNA in the direction from 5' to 3'. From this RNA as primer, DNA polymerase synthesizes a DNA strand in the direction from 5' to 3' on the lagging strand as template. The replicated DNA fragment of approximately 200 bases then binds to the single-stranded DNA from which RNA is

removed. In this replication mechanism, several proteins including DNA helicase and helix-destabilizing protein work together to form the replicating machinery. Other proteins involved include DNA topoisomerase (which prevents twisting during the DNA replication), replication initiation proteins and replication termination proteins. The DNA replication mechanism is described in detail in, for example, "Saibou no Bunshiseibutsugaku [Molecular Biology of the Cell]", 3rd Edition, translated by Keiko Nakamura et al., pp.251-262, Kyoikusha, 1996.

[0027] Upon analysis of the nucleotide sequences of the plasmids pRET1100 and pRET1000, they were found to include sequences of AT-rich homologous or analogous repeats and a sequence thought to have a DNA secondary structure, i.e. a nucleotide sequence predicted to be a DNA replication region (a nucleotide sequence region recognized by proteins involved in DNA replication or a region including the DNA replication origin), in the vicinity of the aforementioned orf relating to DNA replication.

[0028] DNA replication requires a DNA replication region and a region coding for a protein involved in DNA replication (hereinafter referred to as "DNA replication-related protein"). According to the present invention it is possible to obtain data relating to the nucleotide sequences of these regions for both plasmids pRET1100 and pRET1000.

[0029] First, the nucleotide sequences set forth as SEQ ID NO: 35-37 were identified as DNA replication regions for plasmid pRET1100. As regions coding for proteins related to DNA replication

there were identified the nucleotide sequences set forth as SEQ ID NO: 1-3 (orf1), the nucleotide sequence set forth as SEQ ID NO: 4 (orf2), the nucleotide sequences set forth as SEQ ID NO: 5-16 (orf3), the nucleotide sequences set forth as SEQ ID NO: 17-21 (orf4), the nucleotide sequences set forth as SEQ ID NO: 22-26 (orf5), the nucleotide sequence set forth as SEQ ID NO: 27 or 28 (orf6), the nucleotide sequence set forth as SEQ ID NO: 29 or 30 (orf7), the nucleotide sequence set forth as SEQ ID NO: 31 or 32 (orf8), and the nucleotide sequence set forth as SEQ ID NO: 33 or 34 (orf9).

[0030] Construction of a plasmid capable of DNA replication from pRET1100 requires that the recombinant plasmid have at least one DNA replication region and at least one coding region (orf) for a DNA replication-related protein. Thus, the (recombinant) plasmids of the invention are characterized by comprising at least one DNA replication region and at least one coding region for a DNA replication-related protein. The coding region for a DNA replication-related protein preferably has a nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 1, 4, 14, 17 and 22.

[0031] The region of the nucleotide sequence set forth as SEQ ID NO: 76 has been suggested as a promoter involved in expression of replication-related proteins, and the plasmids of the invention preferably comprise a promoter region having the nucleotide sequence set forth as SEQ ID NO: 76.

[0032] For plasmid construction, the DNA fragments are appropriately selected based on the aforementioned nucleotide

sequence data. The present invention also encompasses derivatives or functional (DNA-replicating) fragments of the plasmids.

[0033] Next, the nucleotide sequences set forth as SEQ ID NO: 70-72 were identified as DNA replication regions for plasmid pRET1000. As regions coding for proteins related to DNA replication there were identified the nucleotide sequences set forth as SEQ ID NO: 38-41 (orf10), the nucleotide sequence set forth as SEQ ID NO: 42 or 43 (orf11), the nucleotide sequence set forth as SEQ ID NO: 44 (orf12), the nucleotide sequence set forth as SEQ ID NO: 45 or 46 (orf13), the nucleotide sequences set forth as SEQ ID NO: 48-50 (orf14), the nucleotide sequence set forth as SEQ ID NO: 51 or 52 (orf15), the nucleotide sequence set forth as SEQ ID NO: 53 or 54 (orf16), the nucleotide sequence set forth as SEQ ID NO: 55 (orf17), the nucleotide sequences set forth as SEQ ID NO: 56-60 (orf18), the nucleotide sequence set forth as SEQ ID NO: 61 (orf19), the nucleotide sequence set forth as SEQ ID NO: 62 (orf20), and the nucleotide sequences set forth as SEQ ID NO: 63-69 (orf21).

[0034] Construction of a plasmid capable of DNA replication from pRET1000 requires that the recombinant plasmid have at least one DNA replication region and at least one coding region (orf) for a DNA replication-related protein. Thus, the (recombinant) plasmids of the invention are characterized by comprising at least one DNA replication region and at least one coding region for a DNA replication-related protein. The coding region for a DNA replication-related protein preferably has a nucleotide sequence selected from the group consisting of the nucleotide sequences set forth as SEQ ID NO: 40, 42,

44, 45, 53, 55, 56, 61, 62 and 69.

[0035] The regions with the nucleotide sequences set forth as SEQ ID NO: 67 and 47 are homologous with mobilization proteins, and have been implicated in mobilization. The region with the nucleotide sequence set forth as SEQ ID NO: 75 has been implicated in gene expression of mobilization protein and suggested as a recognition site for an expressed protein. Thus, the plasmids of the invention preferably include mobilization protein regions having the nucleotide sequences set forth as SEQ ID NO: 67 and 47, or include a region involved in mobilization having the nucleotide sequence set forth as SEQ ID NO: 75.

[0036] For plasmid construction, the DNA fragments are appropriately selected based on the aforementioned nucleotide sequence data. The present invention also encompasses derivatives or functional (DNA-replicating) fragments of the plasmids.

[0037] The plasmids or DNA fragments of the invention may also contain nucleotide sequences with a substitution, deletion or insertion of one or a plurality of nucleotides in a DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, or a portion thereof, so long as the function of each region is not impaired.

[0038] The shuttle vectors of the invention may be any which comprise a plasmid or DNA fragment having a DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, and a DNA region that is replicable in *E. coli*, and which are replicable in the genus

Rhodococcus and *E. coli*, such as those having the nucleotide sequences set forth as SEQ ID NO: 89 to 100. The shuttle vectors of the invention may also have nucleotide sequences with one or a plurality of nucleotide substitutions, deletions or insertions in the aforementioned nucleotide sequences, so long as they are replicable in the genus *Rhodococcus* and *E. coli*.

[0039] The "plurality" referred to above will differ depending on the type of region, and specifically may be 2-1100, preferably 2-800, more preferably 2-300, even more preferably 2-100, yet more preferably 2-20 and most preferably 2-10.

[0040] As a plasmid or DNA fragment having substantially the same nucleotide sequence as the aforementioned DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, or a portion thereof, there may be mentioned specifically, a nucleotide sequence which hybridizes with a DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, or a portion thereof, under stringent conditions. Here "stringent conditions" are conditions under which specific hybrids are formed and non-specific hybrids are not formed. While it is difficult to precisely quantify the conditions, one example is a set of conditions that permit hybridization of DNA with high homology, such as 80% or greater, preferably 90% or greater or more preferably 95% or greater homology, while not permitting hybridization of DNA with lower homology. More specifically, there may be mentioned hybridization conditions with ordinary Southern

hybridization washing at 60°C, 1xSSC, 0.1% SDS or preferably Southern hybridization washing at 0.1xSSC, 0.1% SDS corresponding salt concentration. When a DNA fragment with a length of approximately 300 bp is used as a portion of the DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, the hybridization washing conditions may be 50°C, 2xSSC, 0.1% SDS.

[0041] The aforementioned plasmid or DNA fragment having substantially the same nucleotide sequence as the aforementioned DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, or a portion thereof, may be obtained by, for example, modification of a DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, or a portion thereof, by site-directed mutagenesis so as to have a substitution, deletion or insertion of nucleotides at a specific site. Such modified DNA may also be obtained by mutation treatment known in the prior art. As mutation treatments there may be mentioned methods of *in vitro* treatment of DNA including a DNA replication region, DNA replication-related protein coding region, promoter region, mobilization protein region or mobilization-related region, or a portion thereof, with hydroxylamine or the like, and methods of treating a microbe possessing the DNA above, such as the genus *Escherichia*, with ultraviolet rays or with a mutagenic agent ordinarily used for mutagenesis such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or EMS.

[0042] Nucleotide substitutions, deletions or insertions as mentioned above include those found in naturally occurring mutants or variants due to differences in *Rhodococcus* strains.

[0043] A shuttle vector of the invention includes a DNA fragment (A) as the aforementioned plasmid or portion thereof, and a DNA region (B) which is replicable in *E. coli*. In some cases it is preferred for the shuttle vector to comprise a DNA region including a drug resistance gene. In the relevant technical field, a "shuttle vector" is a vector which comprises the DNA replication mechanism for two different cell types, and preferably also a drug resistance gene or the like as a selective marker, allowing its auto-replication in the two different cell types. The DNA fragment (A) as the aforementioned plasmid or portion thereof is a DNA region that is replicable in the genus *Rhodococcus*. The DNA region (B) which is replicable in *E. coli* may be a full plasmid or a portion thereof, so long as it can be replicated and amplified in *E. coli*. As such DNA regions that are replicable in *E. coli* there may be used, for example, the plasmids pUC18, pHSG299 and pHSG398.

[0044] When the shuttle vector of the invention includes a drug resistance gene, the preferred ones are ampicillin resistance gene, kanamycin resistance gene and chloramphenol resistance gene, but there are no particular restrictions on the manner of drug so long as the gene is expressed in the genus *Rhodococcus* and *E. coli* as hosts and confers drug resistance to the host cells, in order to allow verification of the presence of plasmids in the two genera based on resistance to the drug. Also, a plurality of such drug resistance genes may be used in

combination.

[0045] The shuttle vector preferably contains multiple cloning sites (multicloning sites), and the cloning sites and drug resistance gene may be induced from, for example, an *E. coli* plasmid. That is, a publicly known *E. coli* plasmid such as one listed above may be cleaved with an appropriate restriction endonuclease and a DNA region containing the cloning sites and drug resistance gene constructed and ligated with another DNA fragment (a DNA region which is replicable in the genus *Rhodococcus*).

[0046] As an illustration, outline of shuttle vector constructions is shown in Figs. 3 to 5. The shuttle vectors may be constructed by treating the aforementioned plasmids and *E. coli* plasmids with suitable restriction endonucleases and then ligating them. In this manner, the present inventors constructed 18 shuttle vectors (Table 5) using the *Rhodococcus* plasmids pRET1000, pRET1100 or pRET1200, and the *E. coli* plasmids pUC18, pHSG299 or pHSG398.

[0047] The shuttle vectors of the invention are replicable in the genus *Rhodococcus* and *E. coli* as hosts, and are industrially useful. The *Rhodococcus* and *E. coli* strains transformed by the shuttle vectors of the invention, as well as other microbial transformants, are useful in this way and such transformants are also encompassed by the scope of the invention.

[0048] A vector of the invention is characterized by being constructed using a shuttle vector of the invention. Specifically, it is a vector having target DNA inserted therein which is to be introduced into the shuttle vector of the invention. The DNA to be introduced and

the shuttle vector of the invention are treated with appropriate restriction endonucleases and then ligated them to construct the vector. The vector may then be used to obtain transformants having the desired DNA transferred therein.

[0049] As examples of DNA to be inserted there may be mentioned aminoketone asymmetric reductase genes and coenzyme-regenerating system enzyme genes. Aminoketone asymmetric reductase genes are genes coding for aminoketone asymmetric reductases as described in WO02/070714, and more specifically, DNA coding for a protein comprising the amino acid sequence set forth as SEQ ID NO: 78 (aminoketone asymmetric reductase derived from *R. erythropolis* MAK-34), and particularly DNA comprising the nucleotide sequence set forth as SEQ ID NO: 79. The entirety of the content described in WO02/070714 is incorporated herein by reference.

[0050] An aminoketone asymmetric reductase is any having the properties described in WO02/070714, and includes a protein having the amino acid sequence set forth as SEQ ID NO: 78 of the Sequence Listing, as well as proteins having amino acid sequences obtained by deletion, insertion, substitution or addition of one or more amino acids in the aforementioned amino acid sequence, and exhibiting aminoketone asymmetric reduction activity. Aminoketone asymmetric reduction activity is activity of producing an optically active aminoalcohol represented by general formula (2) above using an α-aminoketone represented by general formula (1) above as the substrate.

[0051] There are no particular restrictions on the methods of deletion, insertion, substitution and addition, and any publicly known

methods may be employed. For example, there may be mentioned the methods described in "Zoku Seikagaku Jikken Kouza 1, Idenshi Kenkyuhou II", edited by the Japanese Biochemical Society, p105 (Hirose, S.), Tokyo Kagaku Dojin (1986); "Shin Seikagaku Jikken Kouza 2, Kakusan III (Recombinant DNA Technology)", edited by the Japanese Biochemical Society, p.233 (Hirose, S.), Tokyo Kagaku Dojin (1992); R.Wu, L. Grossman ed., "Methods in Enzymology", Vol. 154, p.350 & p.367, Academic Press, New York (1987); R.Wu, L. Grossman, ed., "Methods in Enzymology", Vol. 100, p.457 & p.468, Academic Press, New York (1983); J.A. Wells et al., "Gene", Vol. 34, p.315 (1985); T. Grundstroem et al., "Nucleic Acids Res", Vol. 13, p.3305 (1985); J. Taylor et al., "Nucleic Acids Res.", Vol. 13, p.8765 (1985); R.Wu, ed., "Methods in Enzymology", Vol. 155, p.568, Academic Press, New York (1987); and A.R. Oliphant et al., "Gene", Vol.44, p.177 (1986). As specific examples, there may be mentioned the site-directed mutagenesis method (site-specific mutagenesis method) utilizing synthetic oligonucleotides, the Kunkel method, the dNTP[αS] method (Eckstein method), and the region-directed mutagenesis method using sulfurous acid or nitrous acid.

[0052] Sugar chains are attached to the majority of proteins, and substitution of one or a plurality of amino acids can modify the attachment of sugar chains. Thus, the aminoketone asymmetric reductases of the invention also include proteins having the amino acid sequence set forth as SEQ ID NO: 78 of the Sequence Listing and having modifications of sugar chains, so long as they exhibit the aforementioned aminoketone asymmetric reduction activity.

[0053] The aminoketone asymmetric reductases of the invention may also have modifications of their amino acid residues by chemical methods, or their derivatives may be enhanced by modification or partial degradation using peptidase enzymes such as pepsin, chymotrypsin, papain, bromelain, endopeptidase and exopeptidase.

[0054] When the aminoketone asymmetric reductases of the invention are produced by a gene recombinant method, a fusion protein may be expressed and then converted or processed into a protein having biological activity which is substantially equivalent to a natural aminoketone asymmetric reductase either *in vivo* or *ex vivo*. In this case, a fusion production method ordinarily employed for genetic engineering may be used, and the fusion protein may be purified by affinity chromatography or the like, utilizing the fused portion thereof. Modification and enhancement of protein structures may be carried out with reference to "Shin Seikagaku Jikken Kouza 1, Tanpakushitsu VII, Tanpakushitsu Kogaku", edited by the Japanese Biochemical Society, Tokyo Kagaku Dojin (1993), by the methods described therein, the methods described in literature cited therein, or methods which are essentially equivalent thereto.

[0055] The aminoketone asymmetric reductase of the invention may also differ from naturally occurring forms in the identities of one or more of the amino acid residues or in the positions of one or more of the amino acid residues. The present invention also encompasses deletion analogues with deletion of one or more (for example, 1-80, preferably 1-60, more preferably 1-40, even more preferably 1-20 and especially 1-10) amino acid residues, substitution analogues with

substitution of one or more (for example, 1-80, preferably 1-60, more preferably 1-40, even more preferably 1-20 and especially 1-10) amino acid residues or addition analogues with addition of one or more (for example, 1-80, preferably 1-60, more preferably 1-40, even more preferably 1-20 and especially 1-10) amino acid residues peculiar to natural aminoketone asymmetric reductases. Also encompassed are enzymes having the domain structure characteristic of natural aminoketone asymmetric reductases. There may also be mentioned isomers of the aminoketone asymmetric reductases.

[0056] So long as the domain structure characteristic of natural aminoketone asymmetric reductases is maintained, all mutants above are also encompassed among the aminoketone asymmetric reductases of the invention. In addition, it is assumed that enzymes having a primary structural conformation substantially equivalent to natural aminoketone asymmetric reductases of the invention, or a portion thereof, as well as enzymes having biological activity substantially equivalent to natural aminoketone asymmetric reductases, may also be included. Naturally occurring mutants may also be mentioned. The aminoketone asymmetric reductases of the invention may be separated and purified in the manner explained below. The present invention encompasses DNA fragments coding for the aforementioned polypeptides, polypeptides of aminoketone asymmetric reductases having all or some of the natural features, and DNA fragments coding for analogues or derivatives thereof. The nucleotides of the aminoketone asymmetric reductases may be modified (for example, with addition, deletions or substitutions), and such modified forms are

also encompassed by the invention.

[0057] An aminoketone asymmetric reductase gene according to the invention is a nucleic acid coding for any of the aforementioned aminoketone asymmetric reductases. As representative examples there may be mentioned nucleic acid coding for a protein having the amino acid sequence set forth as SEQ ID NO: 78 of the Sequence Listing, and especially nucleic acid having the nucleotide sequence set forth as SEQ ID NO: 79, but since several nucleotide sequences (codons) can code for each amino acid, there exist numerous nucleic acids coding for a protein having the amino acid sequence set forth as SEQ ID NO: 78. Thus, all such nucleic acids are also encompassed among the aminoketone asymmetric reductase genes of the invention. Here, "coding for a protein" means that, when the DNA consists of two strands, one of the two complementary strands has a nucleotide sequence coding for the protein, and therefore the nucleic acids of the invention include nucleic acids comprising nucleotide sequences directly coding for the amino acid sequence set forth as SEQ ID NO: 78 and nucleic acids comprising nucleotide sequences which are complementary thereto. In addition, the aminoketone asymmetric reductase genes of the invention may be nucleic acids which hybridize with nucleic acid comprising a nucleotide sequence complementary to SEQ ID NO: 79 under stringent conditions, and which code for proteins with aminoketone asymmetric reduction activity. Here, "stringent conditions" has the same definition as explained above.

[0058] The coenzyme-regenerating system enzyme gene may be one for various dehydrogenases, specifically, glucose dehydrogenase,

glucose-6-phosphate dehydrogenase, aldehyde dehydrogenases, alcohol dehydrogenases, organic acid dehydrogenases and amino acid dehydrogenases. More specifically, there may be suitably used acetaldehyde dehydrogenase, ethanol dehydrogenase, propanol dehydrogenase, glycerol dehydrogenase, formate dehydrogenase, acetate dehydrogenase, butyrate dehydrogenase, lactate dehydrogenase, maleate dehydrogenase and glutamate dehydrogenase.

[0059] A transformant according to the invention is characterized by comprising the aforementioned vector. The transformant is obtained by introducing the vector into host cells. The vector introduction method may be a publicly known method, such as the calcium phosphate method, lipofection, electroporation, microinjection or the like.

[0060] For example, a transformant of the invention comprising a vector having an aminoketone asymmetric reductase gene inserted therein has aminoketone asymmetric reduction activity, and may be applied for an aminoketone asymmetric reductase production method or optically active aminoalcohol production method as described below.

[0061] The method for production of an aminoketone asymmetric reductase of the invention is characterized by comprising a culturing step in which transformants containing a vector having an aminoketone asymmetric reductase gene inserted therein are cultured in medium which allows growth of the transformants, and a purification step in which the aminoketone asymmetric reductase is purified from the transformants obtained in the culturing step.

[0062] The method for culturing may be a publicly known

method with no particular restrictions so long as it permits growth of the cells used, and ordinarily a liquid medium containing a carbon source, nitrogen source and other nutrients is used. As carbon sources for the medium there may be used any of those that can be utilized by the cells. Specifically, there may be mentioned sugars such as glucose, fructose, sucrose, dextrin, starch and sorbitol, alcohols such as methanol, ethanol and glycerol, organic acids such as fumaric acid, citric acid, acetic acid and propionic acid, and their salts, hydrocarbons such as paraffin, and mixtures thereof. As nitrogen sources there may be used any of those that can be utilized by the cells. Specifically, there may be mentioned ammonium salts of inorganic acids such as ammonium chloride, ammonium sulfate and ammonium phosphate; ammonium salts of organic acids such as ammonium fumarate and ammonium citrate; nitric acid salts such as sodium nitrate and potassium nitrate; and inorganic or organic nitrogenous compounds such as meat extract, yeast extract, malt extract and peptone, as well as mixtures thereof. The medium may also contain appropriately added nutrient sources ordinarily used for culturing, such as inorganic salts, trace metal salts and vitamins. When necessary, there may also be added to the medium substances that promote cell growth and buffering substances effective for maintaining the pH of the medium.

[0063] The culturing of the cells may be carried out under conditions suitable for growth. Specifically, the medium pH may be 3-10, preferably 4-9, and the temperature may be 0-50°C, preferably 20-40°C. The cell culturing may be conducted either under aerobic or anaerobic conditions. The culturing time is preferably 10-150 hours,

but should be appropriately determined for the type of cells used.

[0064] The culture solution of the cells cultured in the manner described above is filtered or centrifuged and the cells are rinsed with water or buffer solution. The rinsed cells are suspended in a suitable amount of buffer solution for disruption of the cells. The method of disruption is not particularly restricted but as examples there may be mentioned mechanical disruption with a mortar, Dynomill, French press, ultrasonic cell disruptor or the like. The aminoketone asymmetric reductase in the cell-free extract obtained by filtration or centrifugation of the solid matter from the cell disruptate is recovered by an ordinary enzyme isolating method.

[0065] There are no particular restrictions on the method for isolation of the enzyme and any publicly known method may be employed, but as examples there may be mentioned purification by salting out such as ammonium sulfate precipitation; gel filtration methods using Sephadex and the like; ion-exchange chromatography methods using carriers with diethylaminoethyl groups or carboxymethyl groups; hydrophobic chromatography using carriers with hydrophobic groups such as butyl, octyl and phenyl; dye gel chromatography methods; electrophoresis methods; dialysis; ultrafiltration methods; affinity chromatography methods; high performance liquid chromatography methods and the like.

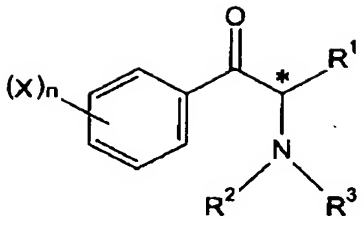
[0066] The enzyme may also be used as an immobilized enzyme. There are no particular restrictions on the method and any publicly known method may be employed, among which there may be mentioned immobilization of the enzyme or the enzyme-producing

cells, and the immobilization may be accomplished by a carrier bonding method such as a covalent bonding method or adsorption method, a crosslinking method, entrapment method or the like. A condensing agent such as glutaraldehyde, hexamethylene diisocyanate or hexamethylene diisothiocyanate may also be used if necessary. Other immobilizing methods include: a monomer method in which a monomer is gelled by polymerizing reaction; a prepolymer method in which molecules larger than monomers are polymerized; a polymer method in which a polymer is gelled; immobilization using polyacrylamide; immobilization using natural polymers such as alginic acid, collagen, gelatin, agar and κ-carrageenan; and immobilization using synthetic polymers such as photosetting resins and urethane polymers.

[0067] The enzyme purified in this manner is judged as having been adequately purified if a single band is confirmed in electrophoresis (SDS-PAGE, etc.).

[0068] A method for production of an optically active aminoalcohol according to the invention is characterized to produce an optically active aminoalcohol compound represented by the following general formula (2), which compound exhibits the desired optical activity, by reacting an aminoketone asymmetric reductase obtained by the production method of the invention with an enantiomeric mixture of an α-aminoketone compound represented by the following general formula (1) or a salt thereof.

[Chemical Formula 8]



... (1)

wherein X may be the same or different and represents at least one species selected from the group consisting of halogen, lower alkyl, hydroxyl optionally protected with a protecting group, nitro and sulfonyl;

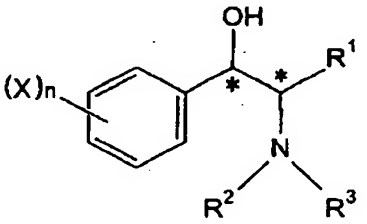
n represents an integer of 0 to 3;

R^1 represents lower alkyl;

R^2 and R^3 may be the same or different and represent at least one species selected from the group consisting of hydrogen and lower alkyl; and

* represents asymmetric carbon.

[Chemical Formula 9]



... (2)

wherein X, n, R^1, R^2, R^3 and * have the same definitions as above.

[0069] First, the α-aminoketone compound represented by general formula (1) according to the invention will be explained.

[0070] The substituent X is as follows. As the aforementioned halogen there may be mentioned fluorine, chlorine, bromine and iodine.

[0071] As lower alkyl there are preferred C1-6 alkyl, among which there may be mentioned methyl, ethyl, propyl, isopropyl, butyl, isobutyl, s-butyl, t-butyl, pentyl, isopentyl, hexyl and the like. These may have straight-chain or branched structures. As substituents they may contain halogens such as fluorine or chlorine, or hydroxyl, alkyl, amino, alkoxy and the like.

[0072] As protecting groups for hydroxyl optionally protected with a protecting group there may be mentioned groups that can be removed by treatment with water, groups that can be removed by acid or weak base treatment, groups that can be removed by hydrogenation or groups that can be removed with Lewis acid catalysts and thiourea, and such protecting groups include optionally substituted acyl, optionally substituted silyl, alkoxyalkyl, optionally substituted lower alkyl, benzyl, p-methoxybenzyl, 2,2,2-trichloroethoxycarbonyl, allyloxycarbonyl, trityl and the like.

[0073] The aforementioned acyl groups include acetyl, chloroacetyl, dichloroacetyl, pivaloyl, benzoyl, p-nitrobenzoyl and the like. They may contain hydroxyl, alkyl, alkoxy, nitro, halogen and the like as substituents. The aforementioned silyl groups include trimethylsilyl, t-butyldimethylsilyl, triarylsilyl and the like. They may contain alkyl, aryl, hydroxyl, alkoxy, nitro, halogen and the like as substituents. The aforementioned alkoxyalkyl groups include methoxymethyl, 2-methoxyethoxymethyl and the like. The aforementioned lower alkyl include C1-6 alkyl, among which there

may be mentioned methyl, ethyl, propyl, isopropyl, butyl, isobutyl, s-butyl, t-butyl, pentyl, isopentyl, hexyl and the like. These may have straight-chain or branched structures. As substituents they may contain halogen such as fluorine or chlorine, or hydroxyl, alkyl, amino, alkoxy and the like.

[0074] X may be nitro or sulfonyl, and specifically there may be mentioned methylsulfonyl and the like.

[0075] The number "n" for X is an integer of 0-3, and is preferably 0.

[0076] R^1 in general formula (1) above represents lower alkyl. As lower alkyl there are preferred C1-6 alkyl, among which there may be mentioned methyl, ethyl, propyl, isopropyl, butyl, isobutyl, s-butyl, t-butyl, pentyl, isopentyl, hexyl and the like. These may have straight-chain or branched structures.

[0077] Each of R^2 and R^3 represent hydrogen or lower alkyl. The lower alkyl include C1-6 alkyl, among which there may be mentioned methyl, ethyl, propyl, isopropyl, butyl, isobutyl, s-butyl, t-butyl, pentyl, isopentyl, hexyl and the like. These may have straight-chain or branched structures.

[0078] As salts of the aforementioned α-aminoketone compounds there may be mentioned salts of inorganic acids such as hydrochloride, sulfate, nitrate, phosphate and carbonate, and salts of organic acids such as acetic acid and citric acid.

[0079] The α-aminoketone can be easily synthesized by halogenation (for example, bromination) of the α-carbon of a corresponding 1-phenylketone derivative, followed by replacement of

the halogen such as bromine with an amine (Ger. (East), 11, 332, Mar.12, 1956).

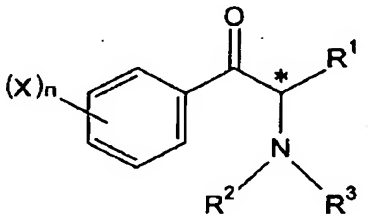
[0080] The optically active aminoalcohol represented by general formula (2) above according to the invention will now be explained. In general formula (2), X, n, R^1, R^2, R^3 and * have the same definitions as in general formula (1) above. As β-aminoalcohols having the desired optical activity there may be mentioned (1S, 2S)aminoalcohols. As specific examples of (1S, 2S)aminoalcohols there may be mentioned d-threo-2-methylamino-1-phenylpropanol (d-pseudoephedrine), d-threo-2-dimethylamino-1-phenylpropanol (d-methylpseudoephedrine), (1S, 2S)-α-(1-aminoethyl)-benzyl alcohol (d-norpseudoephedrine), (1S, 2S)-1-(p-hydroxyphenyl)-2-methylamino-1-propanol, (1S, 2S)-α-(1-aminoethyl)-2,5-dimethoxy-benzyl alcohol, (1S, 2S)-1-(m-hydroxyphenyl)-2-amino-1-propanol, (1S, 2S)-1-(p-hydroxyphenyl)-2-amino-1-propanol, (1S, 2S)-1-phenyl-2-ethylamino-1-propanol, (1S, 2S)-1-phenyl-2-amino-1-butanol, (1S, 2S)-1-phenyl-2-methylamino-1-butanol and the like.

[0081] The conditions for reaction of the aminoketone asymmetric reductase are not particularly restricted so long as an optically active aminoalcohol represented by general formula (2) having the desired optical activity is produced, but since the enzyme optimum pH is 8.1 and the optimum temperature is 55°C, the reaction is preferably carried out under conditions of pH 7-9 and 30-65°C temperature.

[0082] A method for production of an optically active aminoalcohol according to the invention is also characterized to

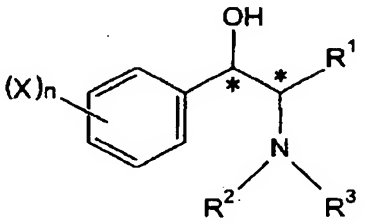
produce an optically active aminoalcohol compound represented by the following general formula (2), which compound exhibits the desired optical activity, by reacting a transformant of the invention with an enantiomeric mixture of an α-aminoketone compound represented by the following general formula (1) or a salt thereof.

[Chemical Formula 10]



... (1)

[Chemical Formula 11]



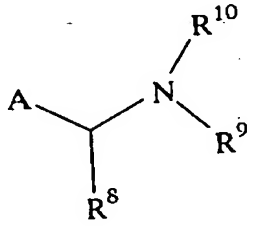
... (2)

[0083] As the reaction conditions for the reaction described above, for example, the transformants shake cultured in liquid medium may be collected, an aqueous aminoketone solution (0.1-10% concentration) added to the obtained cells, and reaction conducted at a temperature of 20-40°C for a period of several hours to one day while regulating the pH to between 6-8. Upon completion of the reaction, the cells may be separated and the product in the reaction solution isolated to obtain an

optically active aminoalcohol. The reaction may be conducted in the same manner for treated transformant cells (dry cells or immobilized cells) or the enzyme or immobilized enzyme obtained from the transformants.

[0084] In the production method for an optically active aminoalcohol of the invention, the reaction may be carried out with further addition of a compound represented by the following general formula (3) or a pharmaceutically acceptable salt or solvate thereof, for more efficient production of the optically active aminoalcohol.

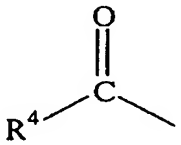
[Chemical Formula 12]



... (3)

(wherein A represents the following formula (Y) or (Z))

[Chemical Formula 13]

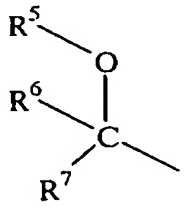


... (Y)

(wherein R^4 represents hydrogen, optionally substituted C1-3 alkyl, a C5-10 hydrocarbon ring which is bonded to R^8 or a 5- to 8-membered

heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^8)

[Chemical Formula 14]



... (Z)

(wherein R^5 represents hydrogen, C1-3 alkyl or a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^6 or R^9, R^6 represents hydrogen, optionally substituted C1-3 alkyl, a C5-10 hydrocarbon ring which is bonded to R^8 or a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^5 or R^9, and R^7 represents hydrogen or optionally substituted C1-6 alkyl); R^8 represents hydrogen, carboxyl, optionally substituted C1-6 alkyl, a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^4 or a C5-10 hydrocarbon ring which is bonded to R^6; R^9 represents hydrogen, optionally substituted C1-6 alkyl, optionally substituted C1-6 alkyloxycarbonyl, optionally substituted acyl or a 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms which is bonded to R^5 or R^6; and R^{10} represents hydrogen or optionally substituted C1-6 alkyl)

[0085] In general formula (3) above, C1-3 alkyl may be straight-chain or branched, and specifically there may be mentioned methyl, ethyl, n-propyl, isopropyl and the like. C1-6 alkyl may be straight-

chain or branched, and specifically there may be mentioned methyl, ethyl, n-propyl, isopropyl, n-butyl, i-butyl, s-butyl, t-butyl, pentyl, hexyl and the like. As C5-10 hydrocarbon rings there may be mentioned cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecanyl and the like.

[0086] As heteroatoms for the 5- to 8-membered heterocyclic skeleton containing 1-3 heteroatoms there may be mentioned nitrogen, oxygen, sulfur and the like, among which nitrogen and oxygen are particularly preferred, and as 5- to 8-membered heterocyclic skeletons there may be mentioned pyrrolidine, piperidine, imidazolidine, piperazine, tetrahydrofuran, tetrahydropyran, tetrahydrothiophene, morpholine and the like.

[0087] As C1-6 alkyloxycarbonyl there may be mentioned methyloxycarbonyl, ethyloxycarbonyl, isopropyloxycarbonyl, isobutyloxycarbonyl, t-butyloxycarbonyl and the like. As acyl there may be mentioned formyl, acetyl, propionyl, butyryl, isobutyryl, pivaloyl, benzoyl, valeryl and the like. When the aforementioned C1-3 or C1-6 alkyl, C1-6 alkyloxycarbonyl or acyl have substituents there are no particular restrictions on the types, positions and numbers of substituents, and as examples of substituents there may be mentioned halogen such as fluorine and chlorine, hydroxyl, alkyl, carboxyl, amino, alkoxy, nitro, aryl and the like. As pharmaceutically acceptable salts there may be mentioned salts of inorganic acids such as hydrochloric acid, sulfuric acid, nitric acid and phosphoric acid, salts of organic acids such as acetic acid and citric acid, salts of inorganic bases such as Na, K, Mg, Ca and ammonia, and salts of organic bases such as

triethylamine and cyclohexylamine.

[0088] As examples of compounds represented by general formula (3) above there may be mentioned 1-acetylamino-2-hydroxypropane, 1-methylamino-2-hydroxypropane, 1-amino-2-oxopropane, 1-amino-2-hydroxycyclopentane, 1-amino-2,3-dihydroxypropane, L-threonine, 4-amino-3-hydroxybutanoic acid, 1-amino-2-oxocyclohexane, morpholine, 3-hydroxypyrrolidine, 3-hydroxypiperidine, 2-aminomethyl-tetrahydrofuran, 1-(2-hydroxypropyl)amino-2-hydroxypropane, 1-t-butyloxycarbonylamino-2-hydroxypropane, 2-amino-3-hydroxybutane, DL-serine, 1-amino-2-hydroxypropane, 1-amino-2-hydroxybutane and 1-amino-2-hydroxycyclohexane. Compounds among these having asymmetric carbons may be optically active forms or racemic forms, unless otherwise specified.

[0089] Addition of such activity inducers to the medium can induce cellular activity and thus more efficiently promote production of the optically active β-aminoalcohol than when no such activity inducers are added. The activity inducers may be used alone, or several such activity inducers may be used in admixture. The amount of such activity inducers is preferably 0.01-10 wt% with respect to the medium.

[0090] The reaction method for production of the β-aminoalcohol of the invention is not particularly restricted so long as it is a method in which the cells or the cell-produced enzyme is reacted with an enantiomeric mixture of an α-aminoketone compound represented by general formula (1) above or its salt, to produce the corresponding

optically active β-aminoalcohol compound represented by general formula (2), and the reaction is initiated by mixing the cells rinsed with buffer solution or water with the α-aminoketone aqueous solution used as the starting material.

[0091] The reaction conditions may be selected within a range that does not impede production of the optically active β-aminoalcohol compound represented by general formula (2). The cell volume is preferably 1/100 to 1000-fold and more preferably 1/10 to 100-fold in terms of dry weight with respect to the racemic aminoketone. The concentration of the racemic aminoketone substrate is preferably 0.01-20% and more preferably 0.1-10%. The pH of the reaction solution is preferably 5-9 and more preferably 6-8, and the reaction temperature is preferably 10-50°C and more preferably 20-40°C. The reaction time is preferably 5-150 hours, but this may be appropriately determined depending on the cell type.

[0092] In order to more efficiently promote the reaction, there may be added sugars such as glucose, organic acids such as acetic acid and energy sources such as glycerol. These may be used alone or as mixtures. The amount of addition is preferably 1/100 to 10-fold with respect to the substrate. Coenzymes and the like may also be added. As coenzymes there may be used nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the like, either alone or in mixtures, added in amounts of preferably 1/1000 to 1/5 with respect to the racemic aminoketone. In addition to such

coenzymes, there may be added coenzyme-regenerating enzymes such as glucose dehydrogenase, in amounts of 1/1000 to 1/5 with respect to the racemic aminoketone. Also, substrates for coenzyme-regenerating enzymes, such as glucose, may be added, in amounts of 1/100 to 10-fold with respect to the racemic aminoketone. There may also be used combinations of sugars such as glucose, organic acids such as acetic acid, energy sources such as glycerol, coenzymes, coenzyme-regenerating enzymes and coenzyme-regenerating enzyme substrates. These usually accumulate in the cells but if necessary they may be added to increase the reaction speed or yield, and therefore may be added as appropriate.

[0093] If the reaction solution is reacted with addition of the specific salts described above under the aforementioned conditions, racemization of the unreacted α-aminoketone isomers will be aided, thus more efficiently promoting conversion to the enantiomer which will serve as the substrate of the cells or cell-produced enzyme. This will tend to yield the target aminoalcohol from the starting material at a high yield of 50% or greater.

[0094] As salts that promote racemization of unreacted α-aminoketones there may be used weak acid salts such as acetate, tartarate, benzoate, citrate, malonate, phosphate, carbonate, paranitrophenol salt, sulfite and borate, but there are preferably used phosphate (for example, sodium dihydrogen phosphate, potassium dihydrogen phosphate, ammonium dihydrogen phosphate), carbonate (for example, sodium carbonate, sodium hydrogen carbonate, potassium carbonate, ammonium carbonate) and citrate (for example,

sodium citrate, potassium citrate, ammonium citrate). Mixtures thereof may also be used, with a buffer solution with a pH of 6.0-8.0 added to a final concentration of preferably 0.01-1 M. In the case of a phosphate, for example, sodium dihydrogen phosphate and sodium monohydrogen phosphate may be mixed in a proportion of between 9:1 and 5:95.

[0095] The optically active α-aminoalcohol produced by the reaction may be purified by ordinary separation and purification means. For example, the optically active β-aminoalcohol may be obtained directly from the reaction solution or after separation of the cells, by being subjected to a common purification process such as membrane separation, extraction with an organic solvent (for example, toluene, chloroform, etc.), column chromatography, vacuum concentration, distillation, crystallization, recrystallization or the like. The optical purity of the produced optically active β-aminoalcohol can be measured by high performance liquid chromatography (HPLC).

Examples

[0096] The present invention will now be explained in greater detail through examples, with the understanding that these examples in no way limit the technical scope of the invention.

[0097] (Example 1) Isolation and purification of plasmids

(1) Method

Rhodococcus strains were inoculated to 5 mL of GPY medium (1% glucose, 0.5% bactopeptone, 0.3% yeast extract) and cultured with shaking at 25°C. After adding 250 μL of a 100 mg/mL ampicillin solution in the logarithmic growth phase, culturing was continued at 25°C for 2 hours with shaking. The cells were harvested by

centrifugation (12 krpm, 5 min), and after removing off the supernatant, they were suspended in 1 mL of 50 mM Tris (pH 7.5), the cells were again harvested by centrifugation (12 krpm, 5 min) and the supernatant was removed off. They were then suspended in 250 μL of a 10 mg/mL lysozyme solution dissolved in TE solution (10 mM Tris (pH 7.5), 1 mM EDTA), and the suspension was allowed to stand at 37°C for 30 minutes. Next, 100 μL of 3 M sodium chloride and 25 μL of 10% SDS were added and the mixture was allowed to stand at -20°C overnight. To the supernatant from centrifugation (12 krpm, 5 min) there were added 0.5 μL each of 50 μg/mL Proteinase K and 50 μg/mL RNase A, and the mixture was allowed to stand at 37°C for 15 minutes. An equivalent of phenol/chloroform/isoamyl alcohol solution was added and centrifugation was performed (12 krpm, 5 min). A 2.5-fold amount of ethanol was added to the supernatant, the mixture was centrifuged (12 krpm, 5 min), and the precipitate was dissolved in 50 μL of sterilized water. Confirmation of plasmids was accomplished by electrophoresis with 0.8% agarose gel and staining with ethidium bromide, followed by UV irradiation.

[0098] (2) Test bacteria strains and results

Throughout the examples, the presence or absence of plasmids was screened from available strains belonging to the genus *Rhodococcus* and its related genus *Mycobacterium* followed the method described in (1) above.

[0099] Table 3 shows the screened strains confirmed to contain plasmids. Specifically, *Rhodococcus erythropolis* (IAM1400, IAM1503, JCM2893, JCM2894 and JCM2895) and *Rhodococcus*

rhodnii (JCM3203) were confirmed to contain plasmids of approximately 5.4 kbp and 5.8 kbp, respectively. These plasmids were designated according to the names listed in Table 3: pRET1100, pRET1200, pRET1300, pRET1400, pRET1500, pRET1600, pRET1700, pRET1800, pRET0500, pRET1000 (see Table 3).

[0100] *R. erythropolis* IAM1400 and IAM1503 are described in "IAM Catalogue of Strains, Third Edition, 2004" published by the Institute of Molecular and Cellular Biosciences, The University of Tokyo, and are available from the institute. Also, *R. erythropolis* JCM2893, JCM2894 and JCM2895 and *R. rhodnii* JCM3203 are described in "JCM Catalogue of Strains, Eighth Edition 2002" published by RIKEN, Japan, and are available from the institute.

[0101] [Table 3]

Strain	No.	Size (kbp)	Name
Rhodococcus erythropolis	IAM 1400	5.4	pRET1100
		5.4	pRET1200
ditto	IAM 1503	5.4	pRET1300
		5.4	pRET1400
ditto	JCM 2893	5.4	pRET1500
		5.4	pRET1600
ditto	JCM 2894	5.4	pRET1700
		5.4	pRET1800
ditto	JCM 2895	5.4	pRET0500
Rhodococcus rhodnii	JCM 3203	5.8	pRET1000

[0102] (Example 2) Identification of restriction endonuclease sites

Various restriction endonucleases were used to determine restriction endonuclease sites, for classification of the plasmids shown in Table 3. Each plasmid was isolated by the method described in

Example 1, and then digested with *EcoR* I, *Hin*d III, *Pvu* II, *Sca* I, *Sph* I, *Sma* I, *Sac* I, *Bam*H I and *Kpn* I, and electrophoresed on 0.8% agarose gel for confirmation of the DNA fragments. The size marker used was Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest (Toyobo). The numbers of sites cleaved by the restriction endonucleases and the sizes of the fragments were determined based on the size marker. The results are shown in Table 4.

[0103] [Table 4]

	R. erythropolis									*R. rhodnii*
	IAM 1400		IAM 1503		JCM 2893		JCM 2894		JCM 2895	JCM 3203
	pRET1100	pRET1200	pRET 1300	pRET 1400	pRET 1500	pRET 1600	pRET 1700	pRET 1800	pRET 0500	pRET1000
*Bam*H I	2(0.4, 5.0)	1(5.4)	same as pRET 1100	same as pRET 1200	same as pRET 1100	same as pRET 1200	same as pRET 1100	same as pRET 1200	same as pRET 1200	2(2.0, 3.8)
*Eco*R I	2(0.3, 5.1)	1(5.4)								0
*Hin*d III	0	0								0
Kpn I	1(5.4)	0								0
Pvu II	1(5.4)	2(0.9, 4.5)								4(0.1, 1.4, 2.0, 2.3)
Sac I	1(5.4)	1(5.4)								3(0.9, 1.0, 3.9)
Sca I	0	0								0
Sph I	0	0								0
Sma I	1(5.4)	2(0.4, 0.5)								4(0.1, 1.2, 1.6, 2.9)

Values in parentheses indicate sizes (kbp)

[0104] Based on the analysis results shown above, the plasmids in Table 3 were classified into three types: plasmids possessing the same restriction endonuclease sites as pRET1100, plasmids possessing the same restriction endonuclease sites as pRET1200, and pRET1000.

[0105] (Example 3) Plasmid sequencing and homology search

As the plasmids were classified into three types, i.e. pRET1000, pRET1100 and pRET1200 based on the results of Example 2, it was attempted to sequence each of the plasmids.

[0106] First, the DNA fragments of the plasmids were cloned for determination of the nucleotide sequences. For *Rhodococcus*

erythropolis (IAM1400), the plasmids (pRET1100, pRET1200) were isolated and digested with *Sma* I and *Sac* I. Upon electrophoresis on 0.8% agarose gel, DNA fragments with sizes of approximately 0.5 kbp, approximately 1.7 kbp, approximately 3.7 kbp and approximately 4.9 kbp were confirmed. The respective DNA fragments were recovered from the agarose gel using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience) and used as insert DNA. Separately, pBluescript II KS(-) was used after digesting with *Sma* I alone or with *Sma* I and *Sac* I, as vector DNA. The insert DNA and vector DNA were ligated with Ligation High (Toyobo) and used to transform *E. coli* JM109. The obtained transformants were screened using a GFX Micro Plasmid Prep Kit (Amersham Bioscience) to obtain different clones.

[0107] For *Rhodococcus rhodnii* (JCM3203), the plasmid (pRET1000) was isolated and then digested with *Bam*H I. Upon electrophoresis on 0.8% agarose gel, DNA fragments with sizes of approximately 2.0 kbp and approximately 3.8 kbp were confirmed. The respective DNA fragments were recovered from the gel using the aforementioned Kit and used as insert DNA. The vector DNA used was pBluescript II KS(-) digested with *Bam*H I.

[0108] Determination of the nucleotide sequences of the plasmid inserts was accomplished by the primer walking method. The apparatus used was an ABI PRISM™ 310NT Genetic Analyzer, and the enzyme used was a BigDye Terminator v3.1 Cycle Sequencing Kit (ABI).

[0109] First, P7 (M13 forward, Toyobo) and P8 (M13 reverse,

Toyobo) primers were used for partial decoding of the insert nucleotide sequences. Next, primers were designed within the decoded sequence (using the sequence analyzing software DNASIS Pro; Hitachi Software Corp.), and the designed primers (synthetic oligo DNA) were used for further decoding of the nucleotide sequence. This procedure was repeated until decoding of the entirety of each insert nucleotide sequence. Upon completion of the insert nucleotide sequence decoding, primers were designed for reaction from the ends of each insert to the vector direction in order to analyze how the inserts were linked, and PCR was conducted (using KOD -plus-), using the plasmid isolated from *Rhodococcus erythropolis* (IAM1400) as template. The PCR product was purified using a GFX™ PCR DNA and Gel Band Purification Kit, and sequencing was carried out using the same primers used for PCR, to analyze the arrangement of the inserts.

[0110] The results of sequencing showed that pRET1100 consisted of 5444 bp, with a G+C content of 59%. The full determined nucleotide sequence is set forth as SEQ ID NO: 73 of the Sequence Listing. Plasmid pRET1200 consisted of 5421 bp and had a G+C content of 62%. Plasmid pRET1000 consisted of 5813 bp and had a G+C content of 67%. The full determined nucleotide sequence is set forth as SEQ ID NO: 74 of the Sequence Listing.

[0111] A homology search for the determined nucleotide sequences using DNASIS Pro revealed that pRET1000 and pRET1100 were novel plasmids. On the other hand, pRET1200 had approximately 99.6% homology with pN30 (GenBank accession no. AF312210) (calculated based on pRET1200).

[0112] For pRET1000 and pRET1100, comparison was made with publicly known plasmids based on the determined nucleotide sequences, using DNASIS Pro. As a result, neither of the plasmids were found to have completely matching restriction endonuclease sites with other plasmids.

[0113] (Example 4) Nucleotide sequence analysis

The results of analysis of the nucleotide sequences of pRET1100 and pRET1000 are shown below.

[0114] The following orfs were found in pRET1100:

orf1 (SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3) consisting of the nucleotide sequence from bases 202, 238 or 337 to 480 of the nucleotide sequence set forth as SEQ ID NO: 73;

orf2 (SEQ ID NO: 4) consisting of the nucleotide sequence from bases 477 to 758 of the nucleotide sequence set forth as SEQ ID NO: 73;

orf3 (SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15 or SEQ ID NO: 16) consisting of the nucleotide sequence from bases 862, 1294, 1450, 1462, 1486, 1489, 1513, 1630, 1645, 1687, 2224 or 2227 to 2409 of the nucleotide sequence set forth as SEQ ID NO: 73;

orf4 (SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20 or SEQ ID NO: 21) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 1875, 1734, 1701, 1674 or 1581 to 1444 of the nucleotide sequence set forth as SEQ ID NO: 73;

orf5 (SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 or SEQ ID NO: 26) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 2828, 2792, 2747, 2594 or 2540 to 2406 of the nucleotide sequence set forth as SEQ ID NO: 73;

orf6 (SEQ ID NO: 27 or SEQ ID NO: 28) consisting of the nucleotide sequence from bases 2971 or 3049 to 3306 of the nucleotide sequence set forth as SEQ ID NO: 73;

orf7 (SEQ ID NO: 29 or SEQ ID NO: 30) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 3577 or 3571 to 3053 of the nucleotide sequence set forth as SEQ ID NO: 73;

orf8 (SEQ ID NO: 31 or SEQ ID NO: 32) consisting of the nucleotide sequence from bases 3339 or 3648 to 3902 of the nucleotide sequence set forth as SEQ ID NO: 73; and

orf9 (SEQ ID NO: 33 or SEQ ID NO: 34) consisting of the nucleotide sequence from bases 4366 or 4477 to 5034 of the nucleotide sequence set forth as SEQ ID NO: 73.

[0115] The following orfs were found in pRET1000:

orf10 (SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40 or SEQ ID NO: 41) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 3350, 3251, 2945 or 2849 to 2412 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf11 (SEQ ID NO: 42 or SEQ ID NO: 43) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 2365 or 2332 to 2159 of the nucleotide sequence set forth as SEQ

ID NO: 74;

orf12 (SEQ ID NO: 44) consisting of the nucleotide sequence from bases 3197 to 3526 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf13 (SEQ ID NO: 45 or SEQ ID NO: 46) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 4035 or 3996 to 3679 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf14 (SEQ ID NO: 48, SEQ ID NO: 49 or SEQ ID NO: 50) consisting of the nucleotide sequence from bases 4621, 4654 or 4666 to 4830 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf15 (SEQ ID NO: 51 or SEQ ID NO: 52) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 5161 or 5062 to 4709 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf16 (SEQ ID NO: 53 or SEQ ID NO: 54) consisting of the nucleotide sequence from bases 2331 or 2334 to 2618 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf17 (SEQ ID NO: 55) consisting of the nucleotide sequence from bases 2907 to 3242 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf18 (SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59 or SEQ ID NO: 60) consisting of the nucleotide sequence from bases 1650, 1689, 1713, 1827 or 1875 to 2162 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf19 (SEQ ID NO: 61) consisting of the nucleotide sequence

from bases 1906 to 2169 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf20 (SEQ ID NO: 62) consisting of the nucleotide sequence complementary to the nucleotide sequence from bases 810 to 553 of the nucleotide sequence set forth as SEQ ID NO: 74;

orf21 (SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68 or SEQ ID NO: 69) consisting of the nucleotide sequence from bases 117, 147, 306, 456, 5144, 5276 or 5534 to 656 of the nucleotide sequence set forth as SEQ ID NO: 74.

[0116] The DNA replication region of pRET1100 is the region represented by the nucleotide sequence set forth as SEQ ID NO: 35 (from bases 2410 to 3200), the nucleotide sequence set forth as SEQ ID NO: 36 (from bases 1000 to 1500) or the nucleotide sequence set forth as SEQ ID NO: 37 (from bases 5000 to 500). The DNA replication region of pRET1000 is the region represented by the nucleotide sequence set forth as SEQ ID NO: 70 (from bases 3355 to 3507), the nucleotide sequence set forth as SEQ ID NO: 71 (from bases 4290 to 4350) or the nucleotide sequence set forth as SEQ ID NO: 72 (from bases 3570 to 3894).

[0117] The region of the nucleotide sequence from bases 5144 to 656 (SEQ ID NO: 67) and the region of the nucleotide sequence from bases 4381 to 4830 (SEQ ID NO: 47) of the nucleotide sequence of pRET1000 (SEQ ID NO: 74) are homologous with mobilization proteins, suggesting that they are involved in mobilization.

[0118] A DNA secondary structure is predicted for the region of

the nucleotide sequence from bases 4260 to 4339 (SEQ ID NO: 75) of the nucleotide sequence of pRET1000 (SEQ ID NO: 74), and it is presumably involved in expression of the mobilization protein gene or is the recognition site of the expressed protein.

[0119] On the other hand, it was suggested that the region of the nucleotide sequence from bases 761 to 868 (SEQ ID NO: 76) of the nucleotide sequence of pRET1100 (SEQ ID NO: 73) is a promoter involved in expression of a protein related to replication.

[0120] (Example 5) Construction of shuttle vectors

For construction of a shuttle vector between *Rhodococcus* strains and *E. coli*, the *Rhodococcus* plasmids pRET1000, pRET1100 and pRET1200 and the *E. coli* plasmids pUC18, pHSG299 and pHSG398 were used for the following experiment.

[0121] First, DNA fragments were prepared from *R. erythropolis* plasmids. Specifically, plasmids pRET1100 and pRET1200 were obtained from *R. erythropolis* (IAM1400), and then *Alw*44 I was used for digestion of pRET1100 at 37°C for 2 hours and Blunting High (Toyobo) was used for blunting of the ends, while *Bsp*LU11 I was used for digestion of pRET1200 at 48°C for 2 hours and Blunting High (Toyobo) was used for blunting of the ends, to obtain DNA fragments of *R. erythropolis* plasmid. Each of the DNA fragments was dissolved in TE solution.

[0122] For pRET1000, plasmid pRET1000 was obtained from *R. rhodnii* (JCM3203), and then *Drd* I was used for digestion of pRET1000 at 37°C for 2 hours and Blunting High was used for blunting of the ends, to obtain pRET1000 DNA fragments, which were

dissolved in TE solution.

[0123] Next, DNA fragments were prepared from the *E. coli* plasmids. Specifically, pUC18 (containing the ampicillin-resistance gene (Amp^r)) was digested with *Sma* I at 30°C for 2 hours, and pHSG299 (containing the kanamycin-resistance gene (Km^r)) and pHSG398 (containing the chloramphenicol-resistance gene (Cm^r)) were digested with *Hin*c II at 37°C for 2 hours to obtain DNA fragments of *E. coli* plasmid, which were dissolved in TE.

[0124] After ligating the DNA fragments from the *Rhodococcus* and *E. coli* plasmids prepared in the manner described above, they were used for transformation in *E. coli* DH5α, which were plated on LB (1% tryptophan, 0.5% yeast extract, 1% sodium chloride; pH 7.2) agar medium containing 100 μg/mL kanamycin, 100 μg/mL ampicillin or 30 μg/mL chloramphenicol, coated with 30 μL of 0.1 M IPTG (isopropyl-β-galactoside) and 4% X-gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside) and allowed to stand at 30°C for 60 hours. White colonies were selected from among the appearing colonies, and were cultured with shaking in LB liquid medium containing 100 μg/mL kanamycin, 100 μg/mL ampicillin or 30 μg/mL chloramphenicol, at 30°C for 60 hours. The DNA was purified from the obtained culture solution using a GFX™ Micro Plasmid Prep Kit (Amersham Bioscience, with purification under the manufacturer's specified conditions). The obtained DNA was confirmed by electrophoresis on 0.8% agarose gel. The obtained shuttle vectors are shown in Table 5, and the methods for constructing each of the shuttle vectors using pRET1100 are shown in Figs. 3 to 5.

[0125] [Table 5]

Constructed shuttle vectors	Origin	
	Rhodococcus	*E. coli*
pRET1001, pRET1001Rv	pRET1000	pUC18
pRET1002, pRET1002Rv	pRET1000	pHSG299
pRET1003, pRET1003Rv	pRET1000	pHSG398
pRET1101, pRET1101Rv	pRET1100	pUC18
pRET1102, pRET1102Rv	pRET1100	pHSG299
pRET1103, pRET1103Rv	pRET1100	pHSG398
pRET1201, pRET1201Rv	pRET1200	pUC18
pRET1202, pRET1202Rv	pRET1200	pHSG299
pRET1203, pRET1203Rv	pRET1200	pHSG398

[0126] The shuttle vectors constructed with pRET1100 and pUC18, pHSG299 or pHSG398 were designated respectively as pRET1101 (SEQ ID NO: 89), pRET1102 (SEQ ID NO: 90) or pRET1103 (SEQ ID NO: 91), respectively. Of the shuttle vectors, pRET1101 exhibits ampicillin resistance, pRET1102 exhibits kanamycin resistance and pRET1103 exhibits chloramphenicol resistance. Also, the shuttle vectors pRET1101 to 1103 wherein the *E. coli* gene and pRET1100 were linked in reverse (Rv) were designated respectively as pRET1101Rv (SEQ ID NO: 92), pRET1102Rv (SEQ ID NO: 93) and pRET1103Rv (SEQ ID NO: 94).

[0127] Similarly, the shuttle vectors constructed using pRET1000 and pRET1200 were designated as pRET1001-pRET1003 (SEQ ID NO: 95-SEQ ID NO: 97) and pRET1001Rv-pRET1003Rv (SEQ ID NO: 98-SEQ ID NO: 100), and as pRET1201-pRET1203 and pRET1201Rv-pRET1203Rv (Table 5).

[0128] (Example 6) Examining method of transformation to *R. erythropolis*

The *Rhodococcus-E. coli* shuttle vectors obtained in Example 5 were used for transformation of *R. erythropolis* MAK-34 strain (MAK-34; deposited at the National Institute of Bioscience and Human-Technology, National Institute of Advanced Industrial Science and Technology, Ministry of Economy, Trade and Industry, (currently: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) on February 15, 2001 as FERM BP-7451). Electroporation was investigated as the method of gene transfer.

[0129] First, *R. erythropolis* MAK-34 strain was inoculated to 5 mL of GPY medium and cultured with shaking at 30°C for 36 hours. After seeding 1 mL of culture solution in 100 mL of LB medium, culturing was continued at 200 rpm at 30°C for 10 hours. The cultured cells were harvested by centrifugation (12 krpm, 5 min, 4°C) and the harvested cells were rinsed twice with ultrapurified water. The rinsed cells were harvested by centrifugation (12 krpm, 5 min, 4°C) and suspended in 2.4 mL of a 10% glycerol solution. The suspension was dispensed into 300 μl portions and frozen at -80°C as competent cells.

[0130] A 90 μL portion of the prepared competent cells and a 5 μL portion of the shuttle vector (pRET1001, pRET1002, pRET1003, pRET1101, pRET1102, pRET1103, pRET1201, pRET1202 or pRET1203) were mixed on ice. The mixed solution was gently poured into a 0.1 cm cuvette which had been cooled on ice, and was set in a Gene Pulser II Electroporation System (BIO-RAD). After pulsing at 20 kV/cm, 400 Ω, 25 μF, the mixed solution was added with 300 μL of LB medium immediately and was allowed to stand at 25°C for 3 hours.

[0131] A portion of the cell suspension was plated on an antibiotic-containing LB plate (100 μg/mL kanamycin, 100 μg/mL ampicillin or 30 μg/mL chloramphenicol). As a result, colonies were obtained when using pRET1002, pRET1102 and pRET1202 containing the kanamycin resistance gene. In order to confirm that the obtained colonies contained the plasmids, the plasmids were isolated and all were verified to contain the shuttle vector.

[0132] This suggested that *R. erythropolis* can be transformed by electroporation and that pRET1002, pRET1102 and pRET1202 function as shuttle vectors.

[0133] (Example 7) Obtaining aminoketone asymmetric reductase gene (*mak* gene)

The *mak* gene was isolated from *R. erythropolis* MAK-34 strain for insertion of the *mak* gene into the shuttle vector shown in Fig. 5.

[0134] First, genomic DNA was obtained from *R. erythropolis* MAK-34 strain. After inoculating *R. erythropolis* MAK-34 strain to 5 mL of GPY medium, culturing with shaking was performed at 30°C for 48 hours, and then the culture solution was seeded in 100 mL of GPY medium and subcultured at 200 rpm at 30°C for 10 hours. The genomic DNA was obtained using a Genomic DNA Buffer set and Genomic-tip 500/G (QIAGEN).

[0135] The obtained genomic DNA was used as template for PCR using KOD -plus-. The primers used were MAKF1 (5'-GAATCTTCTCGTTGATGCAGATCAGGTC-3'; SEQ ID NO: 80) and MAKR2 (5'-CTGACTCCGTAGTGTTCTGCCAGTTC-3'; SEQ ID NO: 81), for PCR at an annealing temperature of 68°C and

extension reaction for 1 minute and 50 seconds. The obtained PCR product was subjected to phenol/chloroform treatment and ethanol precipitation, and then mixed with pUC18 that had been digested with *Sma* I for 2 hours at 30°C, and ligated therewith using Ligation High. Competent High (Toyobo) was used for transformation of *E. coli* DH5α, which was then plated on LB agar medium (containing 100 μg/mL ampicillin) that had been coated with 30 μL of 0.1 M IPTG and 4% X-gal, and was allowed to stand at 30°C for 60 hours. White colonies were selected from among the appearing colonies, and were cultured with shaking in LB liquid medium containing 100 μg/mL ampicillin at 30°C for 60 hours. The DNA was purified from the obtained culture solution using a GFX™ Micro Plasmid Prep Kit. The obtained DNA was confirmed by electrophoresis on 0.8% agarose gel. The obtained clone was designated as pMAK-1.

[0136] (Example 8) Construction of expression vector - 1

A promoter and aminoketone asymmetric reductase gene (*mak* gene) were inserted into the shuttle vector shown in Table 5.

[0137] First, an expression vector (without exogenous promoter) containing approximately 400 bp upstream from the *mak* gene was constructed.

[0138] pMAK-1 was digested with *Sma* I at 30°C for 2 hours, and then with *Pst* I at 37°C for 2 hours. The solution was supplied for 0.8% agarose gel electrophoresis. The DNA size marker used was Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest. After electrophoresis, an approximately 1.4 kbp DNA fragment was purified using a GFX™ PCR DNA and Gel Band Purification Kit, and

used as the insert DNA. On the other hand, the vector used was pRET1102 digested with *Hinc* II and *Pst* I at 37°C for 2 hours. The DNA fragments were ligated with Ligation High and Competent High was used for transformation of *E. coli* DH5α. The cells were plated on LB agar medium containing 100 μg/mL kanamycin and allowed to stand at 30°C for 60 hours.

[0139] The appearing colonies were cultured with shaking on LB liquid medium containing 100 μg/mL kanamycin at 30°C for 60 hours. The DNA was purified from the obtained cultured medium using a GFX™ Micro Plasmid Prep Kit. The obtained DNA was confirmed by 0.8% agarose gel electrophoresis.

[0140] For screening, the obtained DNA without restriction endonuclease treatment and the DNA after digestion with *Pst* I at 37°C for 2 hours were subjected to 0.8% agarose gel electrophoresis, and the target plasmid was obtained based on the size of the DNA. The size marker used was Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest, pRET1102 and pRET1102 that had been digested with *Pst* I at 37°C for 2 hours. The plasmid obtained in this manner was designated as pRET1104.

[0141] (Example 9) Construction of expression vector - 2

The shuttle vectors were reduced, since reduction of shuttle vectors is effective for expression vector enhancement, gene modification, transformation efficiency improvement and replication in cells.

[0142] First, shuttle vector pRET1102 was reduced. After digesting pRET1102 with *Bam*H I and *Hinc* II for 2 hours, it was

electrophoresed on 0.8% agarose gel and an approximately 2.7 kbp DNA fragment was recovered using a GFX™ PCR DNA and Gel Band Purification Kit to prepare a pRET1102 DNA fragment. The size marker used was Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest.

[0143] Separately, a DNA fragment replicable in *E. coli* was prepared by digesting pHSG299 with *Bam*H I and *Hin*c II for 2 hours, subjecting it to 0.8% agarose gel electrophoresis, and recovering an approximately 2.7 kbp DNA fragment using a GFX™ PCR DNA and Gel Band Purification Kit.

[0144] The DNA fragments were ligated with Ligation High and Competent High was used for transformation of *E. coli* JM109 cells, which were then plated on LB agar medium, containing 100 μg/mL kanamycin, that had been coated with 30 μL of 0.1 M IPTG and 4% X-gal, and was allowed to stand at 30°C for 48 hours.

[0145] White colonies were selected from among the appearing colonies, and were cultured with shaking in LB liquid medium containing 100 μg/mL kanamycin at 30°C for 48 hours. The DNA was purified from the obtained culture solution using a GFX™ Micro Plasmid Prep Kit. The reduced shuttle vector of pRET1102 obtained in this manner was designated as pRET1123 (approximately 5.3 kbp).

[0146] Next, shuttle vector pRET1202 was reduced. The *Rhodococcus*-derived DNA fragment was prepared by digesting pRET1202 with *Eco*R I for 2 hours and then with *Dra* III for 2 hours, using Blunting High for blunting of the ends, performing 0.8% agarose gel electrophoresis, and then recovering an approximately 3.7 kbp

DNA fragment using a GFX™ PCR DNA and Gel Band Purification Kit. The size marker used was Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest. The DNA fragment was inserted at the *Hin*c II site of pHSG299. After ligation, Competent High was used for transformation of *E. coli* DH5α, which was then plated on LB agar medium, containing 100 μg/mL kanamycin, that had been coated with 30 μL of 0.1 M IPTG and 4% X-gal, and was allowed to stand at 30°C for 72 hours. White colonies were selected from among the appearing colonies, and were cultured with shaking in LB liquid medium containing 100 μg/mL kanamycin at 30°C for 72 hours. The DNA was purified from the obtained culture solution using a GFX™ Micro Plasmid Prep Kit. When the plasmid obtained by screening was digested with *Sac* I, *Bam*H I, *Pst* I or *Eco*R I for 2 hours, all of the clones had approximately 500 bp clipped at the side of *Eco*R I site of the *Rhodococcus*-derived region. The plasmid was designated as pRET1204 (approximately 5.9 kbp). It was not possible to obtain a clone with no clipping of the genus *Rhodococcus* replication region.

[0147] The shuttle vector pRET1002 was reduced in a similar manner to obtain pRET1006 (approximately 4.9 kbp).

[0148] *R. erythropolis* was transformed with these three reduced plasmids, pRET1006, pRET1123 and pRET1204, and upon confirming the presence or absence of shuttle vector by the method described in Example 6, all the shuttle vectors were detected in the transformed cells. This suggested that the three reduced plasmids pRET1006, pRET1123 and pRET1204 are replicated in *R. erythropolis*.

[0149] (Example 10) Construction of expression vector - 3

An expression vector was constructed by having the mak gene inserted into the shuttle vector constructed in Example 9.

[0150] The *Pst* I site of pRET1123 constructed in Example 9 was deleted for cloning of the promoter in the single step. After digesting pRET1123 with *Pst* I for 2 hours, Blunting High was used for blunting of the ends and Ligation High was used for ligation. The solution was used to transform *E. coli* JM109 using Competent High, and culturing was performed on an LB plate containing 100 μg/mL kanamycin at 30°C for 36 hours. The formed colonies were inoculated on LB liquid medium containing 100 μg/mL kanamycin and cultured at 30°C for 24 hours, and then the DNA was purified using a GFX™ Micro Plasmid Prep Kit to obtain pRET1132.

[0151] The obtained pRET1132 was digested with *Pst* I for 1 hour and then electrophoresed on 0.8% agarose, which resulted in confirming lack of cleavage of pRET1132 by *Pst* I. As controls there were used pRET1123 and pRET1132 not digested with *Pst* I, and pRET1123 digested with *Pst* I.

[0152] (Example 11) Construction of expression vector - 4

A clone was constructed having a promoter and the *mak* gene inserted in the aforementioned shuttle vector.

[0153] A clone was constructed having a *Pst* I site upstream from the *mak* gene, for insertion of a promoter. The procedure was carried out in the following manner to obtain a clone having His-Tag added to the C-terminus of the aminoketone asymmetric reductase. PCR was conducted with KOD -plus- using the pMAK-1 obtained in Example 7 as template, MAKPstF (5'-

GACCACTGCAGATCAATCAACTCTGATGAGGTCC-3'; SEQ ID NO: 82) and MAKHisBglIIR (5'-CGCTTAGATCTCAGTTCGCCGAGCGCCATCGCCG-3'; SEQ ID NO: 83) as primers, with an annealing temperature of 68°C and extension reaction for 1 minute and 50 seconds. A PCR fragment (insert) produced by digesting the obtained PCR product with *Bgl* II at 37°C for 2 hours was ligated with pQE70 (digested with *Sph* I at 37°C for 2 hours, blunted with Blunting High and digested with *Bgl* II at 37°C for 2 hours) using Ligation High, and then Competent High was used for transformation of *E. coli* DH5α cells, which were plated on LB agar medium containing 100 μg/mL ampicillin and allowed to stand at 30°C for 60 hours. The appearing colonies were cultured with shaking on LB liquid medium containing 100 μg/mL ampicillin at 30°C for 60 hours. The DNA was purified using a GFX™ Micro Plasmid Prep Kit. The obtained DNA was confirmed by 0.8% agarose gel electrophoresis.

[0154] For screening, the DNA without restriction endonuclease treatment and the DNA after digestion with *Pst* I and *Bgl* II at 37°C for 2 hours were subjected to 0.8% agarose gel electrophoresis, and the target plasmid was obtained based on the size of the DNA. The plasmid obtained in this manner was designated as pMAK-2. The size marker used was Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest, pQE70, and pQE70 that had been digested with *Bgl* II at 37°C for 2 hours.

[0155] A clone was constructed by inserting the pRET1200 *repA* promoter (obtained by PCR amplification using as template a clone of pRET1204 wherein the orientation of *repA* encoded by the

Rhodococcus-derived DNA fragment was in the same orientation as the kanamycin resistance gene encoded by pHSG299, and using as primers P1200rep-Pst5195 (5'-AGCCGCTGCAGAAGCAACACCGCATCCGCCCATTG-3'; SEQ ID NO: 84) and P7 (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'; SEQ ID NO: 85), with an annealing temperature of 60°C and extension reaction for 1 minute, followed by digestion with *Eco*R I and *Pst* I at 37°C for 2 hours) at the *Eco*R I-*Pst* I site of pMAK-2 (designated as pMAK-19).

[0156] Next, PCR was conducted with KOD -plus- using as template pMAK-19 and as primers pQE70F1 (5'-GGCGTATCACGAGGCCCTTTCGTCTTCACC-3'; SEQ ID NO: 86) and pQE70R1135Bm (5'-GGTTGGATCCGTCATCACCGAAACGCGCGAGGCAG-3'; SEQ ID NO: 87), with an annealing temperature of 60°C and extension reaction for 3 minutes. The PCR product was purified from the reaction solution by using a GFX™ PCR DNA and Gel Band Purification Kit and after digestion of the purified PCR product with *Eco*R I and *Bam*H I for 2 hours, it was electrophoresed on 0.8% agarose gel and the DNA fragment was purified by using a GFX™ PCR DNA and Gel Band Purification Kit. The DNA fragment was used as an insert DNA.

[0157] Separately, a vector to be used as the expression shuttle vector was obtained by digesting pRET1132 with *Eco*R I and *Bam*H I for 2 hours, subjecting the DNA fragment to 0.8% agarose gel electrophoresis and purifying the DNA fragment by using a GFX™

PCR DNA and Gel Band Purification Kit. After mixing the insert DNA and vector, Ligation High was used for ligating them and Competent High was used for transformation of *E. coli* JM109 cells, which were plated on an LB plate containing 100 μg/mL kanamycin. The obtained colonies were cultured on LB liquid medium containing 100 μg/mL kanamycin, and then the plasmid DNA was recovered by using a GFX™ Micro Plasmid Prep Kit and subjected to 0.8% agarose gel electrophoresis for screening. The size markers used were Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest and pRET1132. The obtained expression vector was designated as pRET1133.

[0158] Also, pMAK-19 was digested with *Eco*R I and *Hin*d III at 37°C for 2 hours, blunted with Blunting High and subjected to 0.8% agarose gel electrophoresis, and the approximately 1.6 kbp DNA fragment was purified by using a GFX™ PCR DNA and Gel Band Purification Kit. The clone having this fragment inserted at the *Hin*c II site of pRET1102 was designated as pRET1114.

[0159] The pRET1133 promoter was also modified. The *mak* gene-expressing promoter encoded in pRET1133 is the *repA* gene promoter of pRET1200 and has a length of approximately 800 bp, and a plasmid was constructed by having approximately 200 bp clipped off from this promoter. The promoter used for the cloning was prepared by PCR. Plasmid pRET1200 was used as template, P1204rep-Ec2958 (5'-CGCGGAATTCGACCACCACGCACGCACACCGCAC-3'; SEQ ID NO: 88) and P1200rep-Pst5195 (5'-AGCCGCTGCAGAAGCAACACCGCATCCGCCCATTG-3'; SEQ

ID NO: 84) were used as primers, and KOD -plus- was used as the PCR enzyme for PCR at an annealing temperature of 60°C and extension reaction for 50 seconds. The PCR product was purified by using a GFX™ PCR DNA and Gel Band Purification Kit, digested with the restriction endonucleases *Eco*R I and *Pst* I for 2 hours, and subjected to 1.6% agarose gel electrophoresis, and the DNA fragment was purified by using a GFX™ PCR DNA and Gel Band Purification Kit. The DNA fragment was used as the insert DNA. The nucleotide sequence of the promoter region in the DNA fragment is set forth as SEQ ID NO: 77.

[0160] Separately, for the vector, pRET1133 was digested with restriction endonucleases *Eco*R I and *Pst* I for 2 hours and subjected to 0.8% agarose gel electrophoresis, and an approximately 7.2 kbp DNA fragment was purified by using a GFX™ PCR DNA and Gel Band Purification Kit. The size marker used was Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest.

[0161] The insert DNA and vector obtained in this manner were ligated by using Ligation High, and Competent High was used for transformation of *E. coli* JM109 cells, which were plated on an LB plate containing 100 μg/mL kanamycin. The obtained colonies were cultured on LB liquid medium containing 100 μg/mL kanamycin, and then the plasmid DNA was recovered by using a GFX™ Micro Plasmid Prep Kit and subjected to 0.8% agarose gel electrophoresis for screening. The size markers used were Loading Quick DNA size Marker λ/*Eco*R I+*Hin*d III double digest and pRET1133.

[0162] Also, after digesting the obtained DNA with restriction

endonucleases *Eco*R I and *Pst* I for 2 hours, it was subjected to 1.6% agarose gel electrophoresis and a DNA fragment corresponding to the approximately 600 bp insert DNA was confirmed. The size marker used was a 100 bp DNA Ladder. The expression vector obtained in this manner was designated as pRET1138.

[0163] (Example 12) Preparation of recombinant *R. erythropolis* and measurement of enzyme activity

The aforementioned expression vectors pRET1102, pRET1104, pRET1114 and pRET1138 were used for transformation of *R. erythropolis* MAK-34 strain and *R. erythropolis* JCM2895 (provided by RIKEN Japan), and the enzyme activity was measured. The aminoketone asymmetric reductase purified from MAK-34 strain has the abilities to react with l-2-methylamino-1-phenyl-1-propanone as described in International Patent Publication WO02/070714, and to produce d-(1S, 2S)-pseudoephedrine. It was also reacted with l-2-dimethylaminopropiophenone, 1-amino-2-butanone, etc. and production of each corresponding β-aminoalcohol was confirmed.

[0164] The activity assay was conducted by preparing a reaction solution with a cell density O.D. = 5, 2% glucose and 0.2 M sodium phosphate buffer (pH 6.0), and 3% (1S, 2S)-2-(N-ethylamino)-1-phenyl-1-propanol (EAM) was contained in the reaction as substrate. A synthesis method for EAM is described in J. Am. Chem. Soc., Vol. 50, pp.2287-2292, 1928. The reaction solution was incubated with shaking at 30°C for 16 hours. Confirmation of (1S, 2S)-2-(N-ethylamino)-1-phenyl-1-propanol (EPE), which was β-aminoalcohol as the reaction product, was accomplished by HPLC. The column used

was an Inertsil Ph-3 3.0 × 75 mm, the eluent was aqueous 7% acetonitrile and 0.05 M sodium phosphate buffer (pH 6.0), and the detection was carried out with UV (220 nm).

[0165] The results of the activity assay carried out in this manner are shown in Table 6. The pRET1104-introduced recombinant cells lacking the exogenous promoter region exhibited about the same activity as the pRET1102-introduced recombinant cells lacking the *mak* gene used as the control, and no recombinant enzyme expression was found.

[0166] With transformation of pRET1114 into MAK-34 strain, high specific activity was found compared to pRET1104. This indicated that the pRET1200 *repA* promoter region inserted into the vector functions as a promoter.

[0167] With transformation of pRET1138, the specific activity of the recombinant *R. erythropolis* MAK-34 strain was 37.7 μg/h·mL/O.D. while the specific activity of the recombinant *R. erythropolis* JCM2895 was 34.9 μg/h·mL/O.D., and therefore expression of the enzyme in *R. erythropolis* strain was confirmed.

[0168] [Table 6]

Vector	MAK-34	JCM2895
pRET1102	1.0	1.0
pRET1104	0.7	2.0
pRET1114	17.2	not tested
pRET1138	37.7	34.9

Specific activity (units: μg/h·mL/O.D.)

[0169] (Example 13) Purification of enzyme

The recombinant cells obtained in Example 12 were cultured at

30°C for 4 days in 100 mL of LB medium containing 100 μg/mL kanamycin, the cells were harvested by centrifugation at 12,000 rpm for 5 minutes and the protein having His-tag was purified with The QIAexpressionist Kit (Qiagen). Specifically, the cells were disrupted by ultrasonic treatment, the supernatant was obtained by centrifugation, and the protein was purified with a nickel chelate column. Upon applying the obtained protein to SDS-PAGE, a band of protein, which molecular weight is approximately 28,000, was observed. This molecular weight is roughly equivalent to the molecular weight of the aminoketone asymmetric reductase described in International Patent Publication WO02/070714, thus indicating that the aminoketone asymmetric reductase was produced in the recombinant *Rhodococcus* strains.

[0170] (Example 14) Enzymatic production of β-aminoalcohol

A 0.5 mL portion of reaction solution containing the purified enzyme (0.5 μg/mL) obtained in Example 13, 5 mM NADPH, 120 mM Tris-HCl (pH 7.5) and 5 mM EAM was reacted at 37°C for 16 hours. The substrate and product (EPE) were analyzed by HPLC. The column used was an Inertsil Ph-3 3.0 × 75 mm, the eluent was aqueous 7% acetonitrile and 0.05 M sodium phosphate buffer (pH 6.0), and the detection was carried out with UV (220 nm). The results confirmed production of EPE.

[0171] Similarly, the purified enzyme or the crude enzyme extract obtained from the recombinant cells cultured as described in Example 13 was reacted with l-2-dimethylaminopropiophenone and 1-amino-2-butanone, etc. and production of the corresponding β-aminoalcohols

was confirmed.

Industrial Applicability

[0172] As explained above, the plasmids and shuttle vectors of the invention are derived from *Rhodococcus* strains (especially *Rhodococcus erythropolis* and *Rhodococcus rhodnii*), and when utilized them for modification of the same bacteria by recombination, they allow creation of bacterial strains that more efficiently produce aminoketone asymmetric reductases. They also permit mass production of useful enzymes including aminoketone asymmetric reductases in transformants.